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Transferrin as a fetal growth factor: Acquisition of responsiveness related to embryonic induction

(cell proliferation/tissue interaction/embryonic growth/laminin)

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ABSTRACT **Differentiation of the metanephric mesenchyme, which is triggered by an inductive tissue interaction, has been shown to proceed in a chemically defined medium containing transferrin. Here, we report that neither transferrin-depleted serum nor a chemically defined medium devoid of transferrin promote differentiation and that activity can be restored by the addition of transferrin. It thus appears that we have identified the serum factor required for kidney differentiation. Transferrin seems to affect differentiation by stimulating cell proliferation. We show by using an organ-culture model system that only mesenchymes induced to differentiate by the 24-hr tissue interaction respond to transferrin by proliferation and differentiation, whereas uninduced mesenchymes remain unresponsive. The inductor tissue used is not responsive to transferrin. Thus, the data suggest that the short-range cell-mediated tissue interaction acts by making the nephrogenic mesenchyme responsive to the long-range mediator, which is transferrin. Transferrin is suggested to be an important circulating growth factor required for proliferation during embryogenesis.**

Transferrin is an iron-binding serum protein that has recently received considerable attention because of its profound effect on cell proliferation *in vitro* (1, 2). It is required for the long-term survival of most cells in serum-free media (2), and there is evidence that transferrin stimulates growth by binding to specific proliferation-related cell surface receptors (3–5). We have recently demonstrated that exogenous transferrin promotes differentiation of kidney epithelial cells in an organ culture (6). Since serum contains numerous components thought to be required for differentiation, we have now studied whether transferrin-depleted serum promotes differentiation. The evidence suggests that no other serum factors can replace transferrin, and we report on studies designed to clarify the mechanism of the effect of transferrin on kidney differentiation.

Differentiation of the metanephric kidney is triggered by an inductive tissue interaction between the ureter bud and the nephrogenic mesenchyme (7, 8). In the organ culture model system used here, induction is complete within the first day of culture (9–11). The induced nephrogenic mesenchyme is then converted into an epithelium and, during subsequent days, the polarizing cells express markers for the different segments of the nephron (12–14).

It is known for some other organs that certain serum factors can affect morphogenesis through cell and tissue interactions (15–19). It was therefore considered possible that transferrin could be required for the initial inductive interaction in the kidney model system. The data presented, however, suggest that cells become responsive to transferrin as a result of the initial inductive interaction. Transferrin is then required for proliferation during overt differentiation.

MATERIALS AND METHODS

Culture System. CBA × C57BL hybrid mouse embryos were used. For transfilter cultures, the undifferentiated 11-day metanephrogenic mesenchyme was separated from the ureter bud and placed on a Nuclepore filter. Spinal cord dissected from 11-day embryos was used as a heterotypic inductor (7, 8). The inductor and the mesenchyme were placed on opposite sides of the filter as described (9–11).

Improved minimal essential medium (I-ME medium) was prepared by adding amino acids, vitamins, and minerals but not growth factors or hormones to Eagle's minimal essential medium (20). Human transferrin was used at 50 μg/ml (98% pure, substantially iron free; Sigma) (6). Control cultures were supplemented with 10% fetal calf serum, horse serum (GIBCO), or normal human serum (NHS).

Depletion of Transferrin by Immunoadsorption. Transferrin was removed from NHS by passing 1 ml of NHS through an anti-human transferrin immunoadsorbent column four times. After each pass, the column was washed with phosphate-buffered saline (P_i/NaCl), eluted with 8 M urea, washed with P_i/NaCl, and used again. The immunoadsorbent column was prepared by coupling 20 mg of gamma globulin to 5 ml of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) according to the instructions of the manufacturer. The gamma globulin fraction was prepared from 5 ml of anti-human transferrin serum (Behringwerke) by the $(NH_4)_2SO_4$ precipitation method (14). The depletion of transferrin was checked by the double-immunodiffusion method. The eluate was dialyzed in P_i/NaCl to remove urea before testing, and both the eluate and the adsorbed NHS were concentrated to 1 ml. The anti-human transferrin antiserum gave a strong precipitation line with the original NHS and with the eluate but not with the adsorbed NHS, even after repeated filling of the antigen well (Fig. 1). As judged by these methods, the depleted concentrated serum contained less than 3 μg of transferrin per ml. When tested for differentiation-promoting activity, 10% of this serum was used in the medium (I-ME medium).

Antibodies and Lectins. The rabbit anti-rat kidney brush border antiserum and the rabbit antiserum reacting with Tamm–Horsfall glycoprotein were used to identify proximal and distal tubules (14, 21). Binding of the lectin wheat germ agglutinin to sialic acid moieties was used as a probe to detect the glomerular polyanionic coat (14). Previously described rabbit antibodies to bovine type III procollagen and mouse laminin were used (22, 23).

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Abbreviations: I-ME medium, improved minimal essential medium; NHS, normal human serum; P_i/NaCl, phosphate-buffered saline.

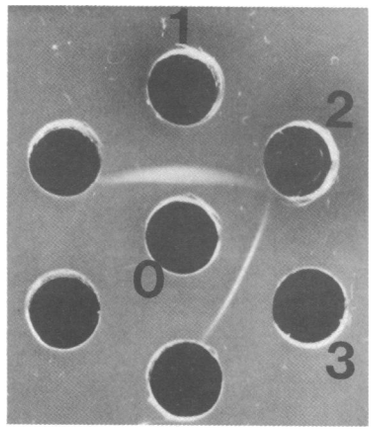

FIG. 1. Immunodiffusion in agarose. NHS (well 1), transferrin-depleted NHS (well 2), and the NHS fraction that bound to the antitransferrin immunoadsorbent column and then eluted with 8 M urea (well 3) were tested against antitransferrin antibodies (well 0).

Fluorescence Microscopy and Histology. Explants prepared for histology or immunohistology were comprised of three to five mesenchymes cultured transfilter with spinal cord. For immunohistology, the tissues were fixed in cold EtOH and processed for fluorescence microscopy (13, 24). For histological examination, tissues were fixed in formalin, embedded in paraffin, and stained with hematoxylin/eosin.

DNA Synthesis. Cell proliferation was monitored by analyzing thymidine incorporation after various times of cultivation. For the 2 last hr of culture, thymidine (specific activity, 18–25 μCi/mmol; 1 Ci = 37 GBq; Radiochemical Centre) was added to the culture medium at a concentration of 25 μCi/ml. The explants were then placed on ice and sonicated in 0.5 ml of distilled water. Samples were taken for the determination of radioactivity in trichloroacetic acid precipitable material (0.2 ml) and for measurement of DNA content (0.225 ml). DNA content was measured microfluorometrically (25). Data are expressed as cpm/μg of DNA. Each point represents the mean of the three explants. Three mesenchymes comprised one explant. An amount of embryonic spinal cord tissue approximately equal to three mesenchymes was analyzed similarly.

RESULTS

Effect of Transferrin on Morphogenesis. Induction of the metanephrogenic mesenchyme in the transfilter model system is completed during the first 24 hr of culture in serum-containing medium (9–11, 14). In this study, we cultured the explants in a chemically defined medium containing transferrin, previously shown to support kidney morphogenesis (6). When the inductor tissue was removed after 24 hr of culture, formation of the different segments of the nephron was seen in the induced mesenchyme after 96 hr. The tubules were surrounded by a basement membrane containing laminin and markers for both proximal and distal tubules could be detected (Fig. 2). Glomerular-like bodies expressing the podocyte polyanionic coat were also found. Our results also show that induction is complete after 24 hr in the chemically defined medium and that transferrin can replace serum in promoting subsequent differentiation in the absence of spinal cord tissue.

We next cultured the transfilter explants in transferrin-free I-ME medium. A shift in the extracellular matrix composition is known to take place during the initial inductive event on day 1 (12, 13). Immunohistology showed that a similar change occurs in explants cultured in a chemically defined medium in both the presence and absence of transferrin. After 24 hr of culture in transferrin-free I-ME medium, the mesenchyme expressed laminin whereas type III procollagen was confined to the upper border of the tissues (Fig. 3). Explants cultured for longer periods in transferrin-free I-ME medium did not, however, continue to differentiate and did not express kidney-specific markers. These results suggest that transferrin is not required for the initial induction (day 1) and that the induced mesenchyme regresses during subsequent culture (days 2–5) unless transferrin is present in the medium.

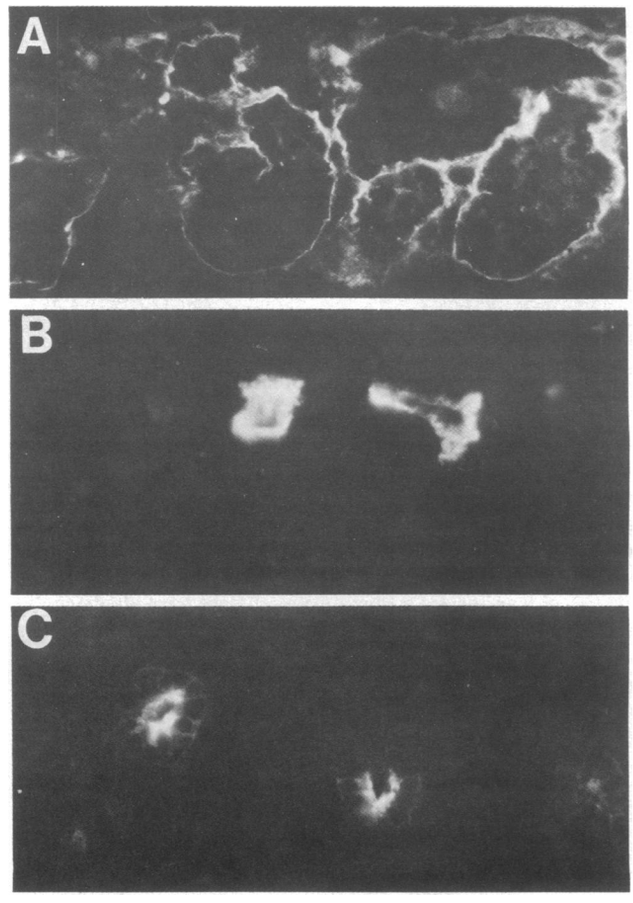

FIG. 2. Immunofluorescence micrographs of explants cultured for 6 days in I-ME medium containing transferrin at 50 μg/ml. Inductor tissue was removed at 24 hr. Laminin is found in the basement membrane area of the formed tubules (*A*). Markers for proximal and distal tubules appear under these conditions, as shown with antibodies to brush border antigens (*B*) and to Tamm–Horsfall glycoprotein (*C*).

Effect of Transferrin on Thymidine Incorporation. Thymidine incorporation in the mesenchymes decreases gradually during the first 16 hr and then increases by 24 hr in our transfilter cultures (26, 27). In those studies, serum-containing culture medium was used. We have now examined thymidine incorporation in defined media with and without transferrin during both induction and differentiation. The initial decrease and the subsequent increase in thymidine incorporation were similar in mesenchymes cultured in I-ME medium with and without transferrin (Fig. 4*A*). This indicates that transferrin does not affect thymidine incorporation during the induction period (0–24 hr). During the subsequent stages, however, thymidine incorporation was dependent on transferrin. In explants cultured in I-ME medium containing transferrin, thymidine incorporation stayed at a high level for several days; in contrast, in explants cultured in transferrin-free I-ME medium, there was a rapid decrease in incorporation on day 2 (Fig. 4*A*). At all stages, transferrin (50 μg/ml) caused a higher increase in thymidine incorporation than 10% horse or fetal calf serum. The effect of transferrin on thymidine incorporation reflected an effect on

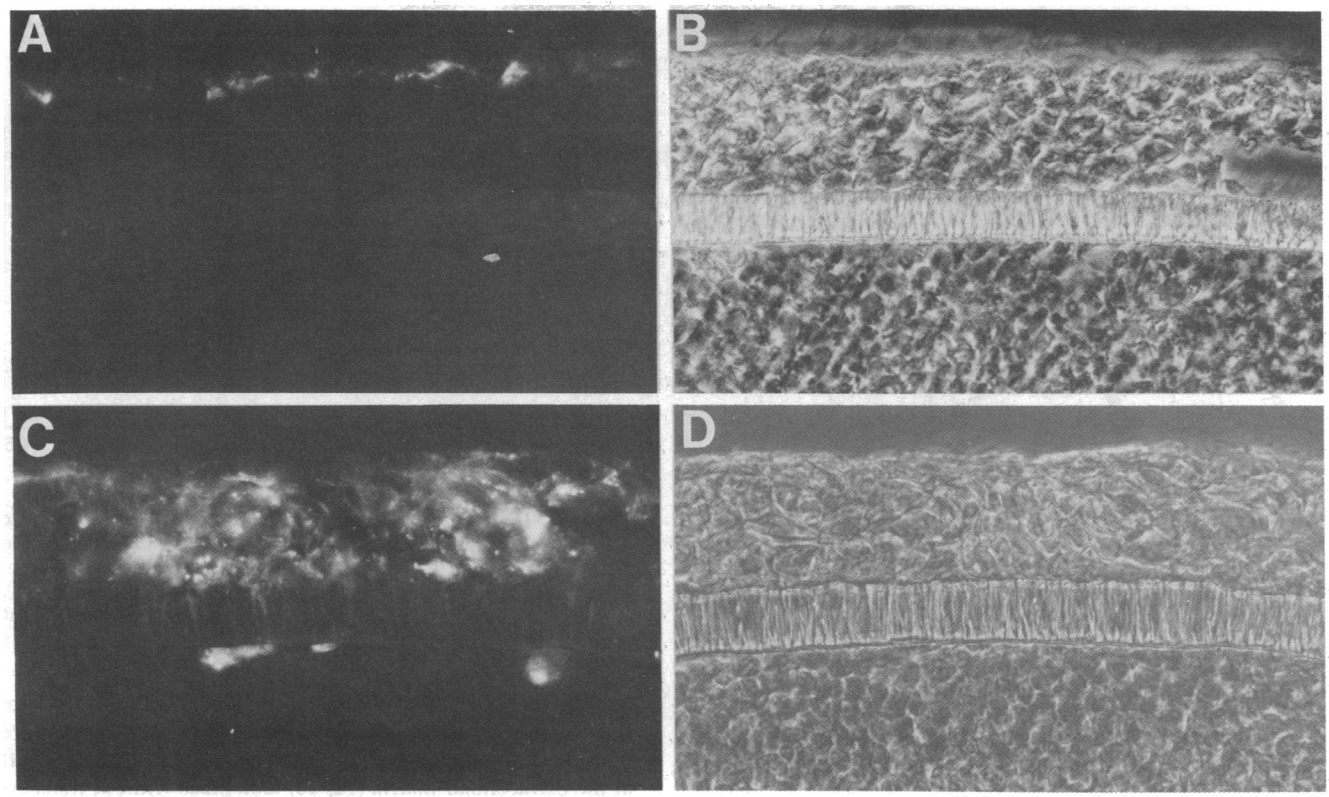

FIG. 3. Micrographs of explants cultured for 24 hr in transferrin-free I-ME medium. (*A* and *C*) Immunofluorescence. (*B* and *D*) The corresponding phase-contrast images. Type III procollagen is found only at the upper surface of the mesenchyme (*A* and *B*), whereas laminin is found throughout the mesenchyme (*C* and *D*).

cell proliferation since the DNA content was constant throughout the culture period, despite earlier findings that substantial amounts of cells are lost during the culture (26). When transferrin was not present, the DNA content gradually decreased (Fig. 5). Although the induced cells did not differentiate or incorporate thymidine in transferrin-free I-ME medium, they survived, since the level of incorporation (≈2,000 cpm/μg of DNA) remained four times that of uninduced cells (≈500 cpm/μg of DNA) (Fig. 4).

We next tested whether the effect of transferrin on DNA synthesis required the presence of an inductor tissue. Isolated noninduced mesenchymes cultured without the inductor tissue did not respond to transferrin at any stage of culture (Fig. 4*B*). The mesenchyme thus could not respond to transferrin by proliferation unless it had been exposed to the inductor. The effect of transferrin was nevertheless not mediated through the inductor tissue. First, DNA synthesis in the inductor tissue was not affected by transferrin. Second, transferrin stimulated incorporation of thymidine in the induced mesenchymes on day 2, even when the inductor tissue was removed at 24 hr. This suggested that the inductive event makes the cells responsive to transferrin. We therefore analyzed whether the appearance of the transferrin responsiveness can be prevented by using small-pore filters that prevent induction (9–11). In such transfilter experiments, thymidine incorporation remained at a low level in spite of the presence of both the inductor tissue and transferrin. The incorporation was similar to that seen in mesenchymes cultured in isolation (Fig. 6).

Effect of Transferrin-Depleted Serum on Morphogenesis. To test whether other components of serum can replace transferrin as a stimulator of differentiation, explants were cultured in I-ME medium supplemented with 10% transferrin-depleted NHS. Such explants failed to differentiate (Fig. 7*A*). In these explants, as well as in 11-day whole kidneys cultured in this medium, cells were very well preserved. When transferrin (50 μg/ml) was added to I-ME medium containing the transferrin-depleted serum, abundant tubule formation was again evident in the explants (Fig. 7*B*). Explants cultured in the defined medium supplemented with transferrin at 50 μg/ml also formed tubules, but the stroma surrounding the tubules was scanty when compared with explants cultured in transferrin-depleted serum supplemented with transferrin at 50 μg/ml. These results show that the additional factors that slightly enhance tubule formation (6) were not removed during the removal of transferrin by immunoadsorption and that no toxic agents were introduced by the adsorption procedure. Thus, the failure to obtain tubules by using the immunoadsorbed serum was due solely to the absence of transferrin.

DISCUSSION

Although it has been known for some time that transferrin is required for cell growth *in vitro* (2), the effect of the substance on embryogenesis has not received much attention, possibly because it is usually classified as a mere nutrient (28). The search for general serum regulators of embryonic growth and differentiation has focused on various hormones such as insulin (28–30), the somatomedins (31–34), and the polypeptides classified as true growth factors (35–37). In this study, we have identified transferrin as the main serum component stimulating kidney differentiation. Experiments with transferrin-depleted serum suggest that other factors in serum cannot replace transferrin. Transferrin is thus a molecularly defined effector in this model system. It was recently shown that the "muscle trophic factor" required for myogenesis (38) is identical to transferrin (39, 40). Evidence from these two developmental systems therefore suggests that transferrin is an important serum factor for organogenesis. The data presented here suggest that transferrin affects differentiation by stimulating cell proliferation.

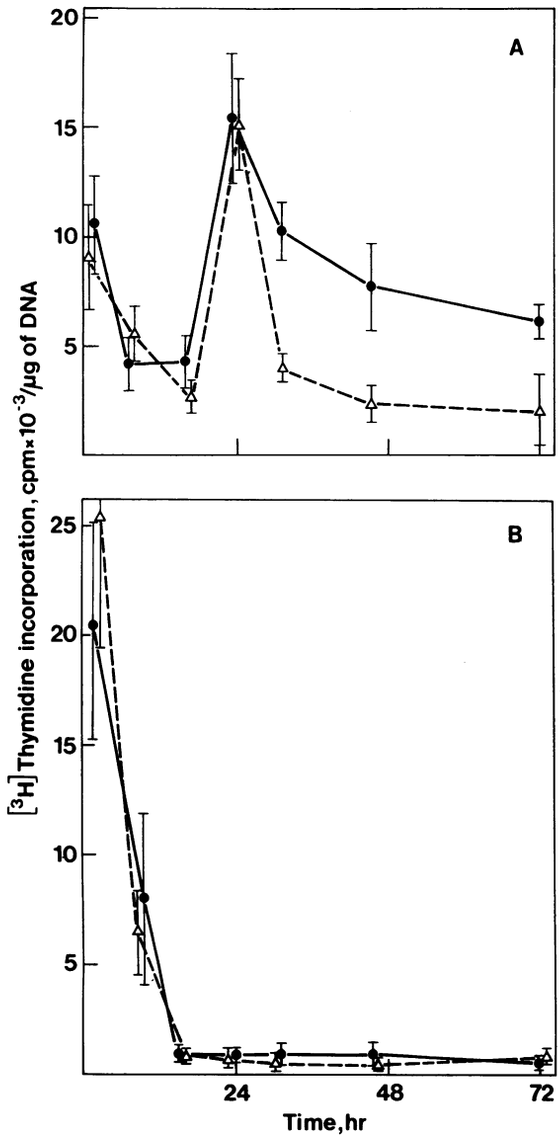

FIG. 4. Time course of thymidine incorporation in DNA showing acquisition of transferrin responsiveness on day 2 of culture *in vitro*. Explants were cultured in I-ME medium in the presence (●) and absence (△) of transferrin at 50 μg/ml. Filter pore size, 1.0 μm. (*A*) Inductor tissue present. During the first day of culture, DNA synthesis is independent of exogenous transferrin but, during later stages, DNA synthesis decreases unless transferrin is present. (*B*) Inductor tissue not present. A rapid decrease in DNA synthesis occurs. No transferrin responsiveness appears. Thymidine incorporation during the first hours is higher than that of mesenchymes cultured transfilter with inductor tissue. The reason for this difference is unknown. Results represent mean ± SD of three explants.

The transfilter model system (8–11) made it possible to identify the mesenchyme as the target tissue for transferrin. However, the mesenchyme remained totally unresponsive unless it was first induced by the inductor tissue. Neither noninduced mesenchyme nor the inductor tissue used responded to transferrin by proliferation. These results suggested that the mesenchyme becomes responsive to transferrin as a consequence of the inductive event. A time-course study of DNA synthesis in the presence and absence of transferrin showed that only induced cells respond to transferrin by proliferation. This responsiveness started on day 2 and then persisted for several days even in the absence of inductor tissue. The induced mesenchyme is therefore the sole target for transferrin, but its response at these later stages is nevertheless dependent on the

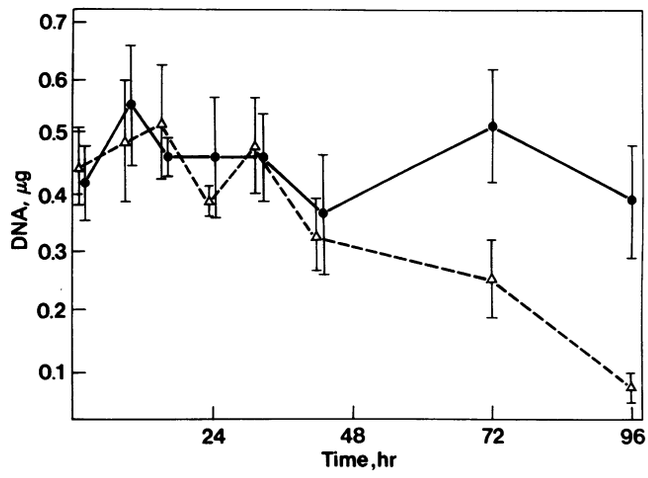

FIG. 5. DNA content of explants containing three metanephric mesenchymes cultured with the inductor tissue as in Fig. 4*A*. In the presence of transferrin at 50 μg/ml (●), the amount of DNA was constant throughout the culture period. In the absence of transferrin (△), the DNA content decreased after day 2 of culture. Results represent mean ± SD of three explants.

presence of the inductor tissue on day 1. The data reported here show that several changes during induction do not require transferrin. Immunocytochemistry showed that the early shift in the extracellular matrix (12, 13) took place without transferrin. DNA synthesis during induction also was unaffected by the addition of transferrin. It is not likely that the early changes are due to production of transferrin by the inductor. First, as shown,

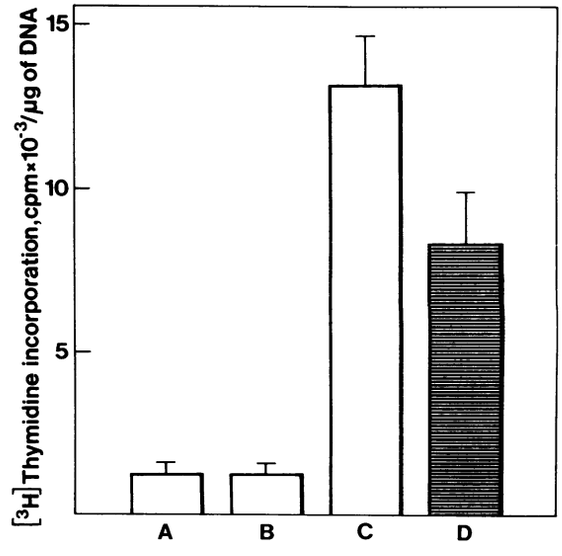

FIG. 6. Requirement of induction of the metanephric mesenchyme for appearance of the transferrin responsiveness. DNA synthesis in the various recombinations was analyzed on day 2. In these experiments, two mesenchymes comprised one explant and the labeling period was extended to 3 hr (42–45 hr of culture). A response is seen when induced mesenchymes are cultured in the presence of transferrin (50 μg/ml) or serum but not when uninduced mesenchymes are cultured in the presence of transferrin. Note that transferrin stimulates DNA synthesis more effectively than 10% serum. Bar A: Mesenchyme cultured alone in I-ME medium containing transferrin. Bar B: Mesenchyme cultured transfilter with inductor tissue using a 0.05-μm Nuclepore filter, which prevents induction (9, 10), in transferrin-containing I-ME medium. Bars C and D: Mesenchyme cultured transfilter with inductor tissue (spinal cord) using 1.0-μm Nuclepore filters in I-ME medium containing transferrin (bar C) or 10% horse serum (bar D). Results represent mean ± SD of three experiments.

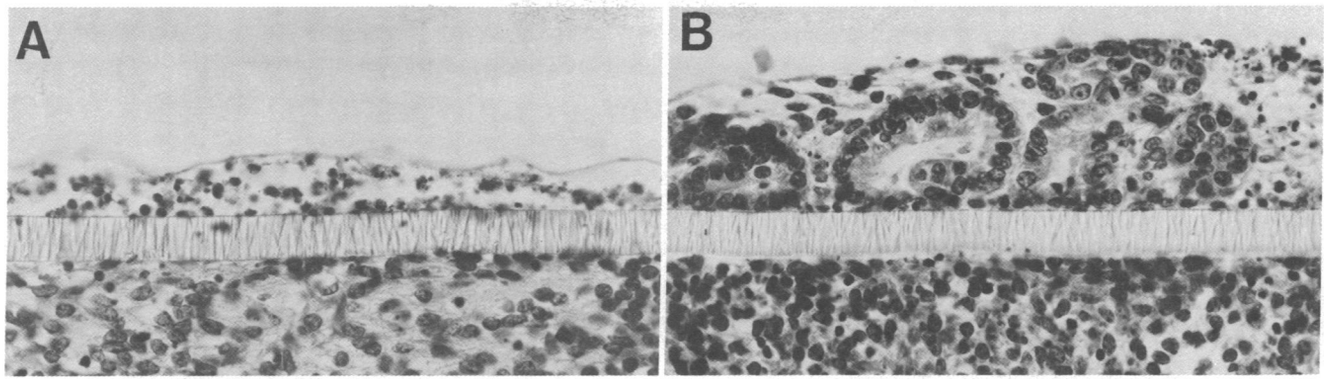

FIG. 7. Histological sections of explants cultured for 4 days. (*A*) Culture in 10% transferrin-depleted NHS. (*B*) Culture in 10% transferrin-depleted NHS supplemented with transferrin at 50 μg/ml. Well-developed tubules are seen only if transferrin is present in the medium. Cells survive well in 10% transferrin-depleted NHS but do not differentiate.

exogenous transferrin is not active on uninduced mesenchymes. Second, we detect transferrin production in 11-day embryonic liver, but using the same technique, we fail to detect transferrin production in the spinal cord (unpublished data).

Our studies, rather, suggest that the inductor tissue acts by making the nephrogenic mesenchyme responsive to transferrin. This is in agreement with proposals that hormone responsiveness during embryogenesis is controlled by tissue interactions (15–19). In the kidney model system, the soluble serum mediator is not a hormone but an iron-binding serum protein and it is active after the cell-mediated inductive interaction.

We do not at present know whether the acquisition of transferrin responsiveness is caused by stimulation of expression of transferrin receptors or whether it is related to the previously described changes in the extracellular matrix composition during induction (12–13). Transferrin-receptor density is known to be dependent on the state of activation of cells (3–5, 41–43). On the other hand, several lines of evidence suggest that, at least in monolayer cultures, cells are more responsive to growth factors when they are attached to a proper extracellular substratum (44–47). Such interactions between the cell, the matrix, and growth factors could be important for differentiation as well (48).

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